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13. ABSTRACT *(Maximum 200 Words)*

Par-4 (prostate apoptosis response protein #4) was first identified as a protein preferentially expressed in rat prostate tissue upon induction of apoptosis. Its effect on expressing cells is to infer super-sensitivity to apoptotic stimuli. Par-4 induction is an early and pivotal response in the apoptotic pathway, and its mode of action has been traced to interaction with zinc-binding domains of three proteins: the atypical PKC isoforms zeta and lambda/iota, and WT1. The interaction between the Par-4 C-terminal region (CTR) and the atypical PKCs has been shown to repress the kinase activity, leading to enhanced cell death. We have succeeded in creating multiple expression plasmids coding for the Par-4 CTR and the PKC-zeta and PKC-lambda/iota Zn-binding domains, and have purified the corresponding polypeptides from *E. coli* hosts. CD spectra and preliminary NMR spectra indicate that the Par-4 CTR is highly helical and self-associating, consistent with its predicted Leucine Zipper structure. Interactions with a native-like fold of the PKC-zeta peptide have been preliminarily confirmed, and studies are underway to prepare for determination of the interaction interface and the structure of the complex to atomic resolution.

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Steven Pascal 10-28-99

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INTRODUCTION

One of the crucial proteins in the apoptotic pathway has been named Par-4, for prostate apoptosis response protein #4(1). This protein, which is specifically induced in certain cells by apoptotic stimuli, sensitizes these cells to apoptosis. It is believed that this sensitization is a result of the influence of Par-4 on a number of other cellular proteins. A putative leucine zipper (LZ) domain near the Par-4 carboxy -terminus (CTR) has been shown to alter the apoptotic effects of at least three proteins. Binding of the Par-4 CTR to the zinc finger (ZF) containing regulatory regions of two atypical isoforms of protein kinase C (aPKC) down-regulates the kinase activity of these proteins(2) (3), which in turn triggers cell death. The Par-4 CTR also binds to the ZF region of the Wilms' tumor suppressor protein WT1(4). WT1 has been shown to block apoptosis at low concentrations, an effect which the Par-4 interaction can reverse. We have proposed to use Nuclear Magnetic Resonance (NMR) spectroscopy and other techniques to determine the three-dimensional structure and oligomerization state of the Par-4 CTR. Also, we had originally proposed to begin structure determination of a complex between pertinent fragments from Par-4 and WT1, but have opted to focus instead on the alternate interaction mentioned in our original proposal, that between Par-4 and the atypical PKCs. An atomic resolution structure of the Par-4 CTR in isolation, and/or in complex with the Zn-binding domains of PKC zeta and/or lambda/iota isoforms will be a natural starting point for structure-based drug design, with the long-term goal of allowing therapeutic control of the apoptotic pathway in androgen-independent prostate cancer cells and other cell types.

BODY

Task 1 Express and purify Par-4 CTR and optimize buffer conditions for NMR studies

a. Express and purify unlabeled Par-4 CTR [month 1-2]

Steven Sells in the Vivek Rangnekar laboratory kindly sent three expression constructs encoding various regions of rat Par-4, including a full length construct (1-332) in pRset B, residues 215-332 in pRset C, and 240-332 in pRset A. Unfortunately, none of the constructs expressed well, despite trials with various E. coli strains, induction temperatures and lengths, and IPTG concentrations. Discovery of a mis-sense mutation in the 240-332 construct prompted us to transfer this insert from pRsetA to pRetC. However, no improvement in expression was seen, and trial purifications using nickel or cobalt beads to bind the six-histidine affinity tag did not yield detectable amounts of protein.

We also were able to obtain a Par-4 280-332 construct in pGex-2TK (a gift from Yang Shi). This construct expressed well, but after sonication of the cells and centrifugation, greater than 95% of the protein was found in the pellet. Treatment with 2M urea successfully resolubulized the pelleted fusion protein, while retaining the ability bind glutathione resin. We will be pursuing the possibility of refolding this fragment.

In an attempt to increase expression levels and solubility, and to avoid a potential secondary thrombin cleavage site between residues 293 and 294, we subcloned residues 215-332 and 240-332 into pGex 6P-1 and pMal-c2. The pRset C 215-332 construct was used as a template. Use of these constructs necessitates Precission protease and Factor Xa, respectively, to cleave the fusion partner from Par-4. All four of these constructs expressed well, producing 10-20 mg of fusion protein per liter of growth. However, ~50% of the protein is in the pellet after sonication. The pMal constructs also produced 10-20 mg fusion protein per liter of growth, with 30% found in the pellet.

The GST-fusions could be purified to 90-95% by glutathione sepharose, and HRV-14 3C protease (3C pro, Pharmacia) could be used to cleave the Par-4 from GST. Since considerable amounts of

several different constructs of the Par-4 CTR will need to be produced in order to optimize solubility and self-associative conditions, we created an expression plasmid for HRV-14 3C protease (3C pro) as a more economical alternative to the Pharmacia product. Home-grown 3C pro successfully and efficiently cleaved the GST-Par-4 fusion (**Figure 1**), and liberated Par-4 fragments were washed from the glutathione resin using 1 M NaCl. Similarly, the pMal fusion proteins were purified on amylose resin, and cut with Factor Xa. However, secondary cuts by Factor Xa were seen, producing two fragments between approximately 9 and 7 kDa as judged by SDS PAGE (**Fig. 1(a), lane 7**). Attempts to optimize Factor Xa cleavage conditions did not significantly reduce degradation. Therefore, we are currently focusing on the pGex constructs.

b. Determine oligomerization state via gel filtration & NMR diffusion [month 2-3]

The Par-4 CTR fragments produced thus far have a tendency to form aggregates in solution when concentrated (see part c below). Oligomerization state will be further examined using various buffer conditions and constructs, and while co-solubilized with the PKC zeta Zn-binding domain, as discussed below.

c. Optimize buffering conditions (pH, buffer, salt, temperature) [month 2-8]

The Par-4 215-332 and 240-332 fragments were washed from glutathione sepharose after 3C pro treatment (the GST remains on the resin), but then precipitated when concentrated to ~ 0.04 mg/ml. We had previously optimized the NaCl concentration to 1 M NaCl, and now also tested various buffer and pH conditions. The peak concentration we were able to obtain without precipitation was 0.55 mg/ml in 12 mM PBS 0.5-1.0 M NaCl, pH 3.5-4.0.

d. Preliminary 1D proton NMR spectra [month 7-8]

We acquired a low concentration (less than 0.05 mg/ml) circular dichroism (CD) spectrum of the 240-332 fragment at pH 7.3, and compared this with CD spectrum of the same fragment after at pH 3.6 and 2.7. As seen in **Figure 2**, all three CD spectra indicate a high degree of alpha helicity, and are nearly identical in shape. Therefore, we believe that the fold does not change significantly upon reduction to acidic pH. A one dimensional NMR spectrum of the 240-332 fragment was acquired at pH 3.5 **(Figure 3(a)).** The peaks are fairly sharp, suggesting a relatively low aggregation state. However, insufficient chemical shift dispersion is seem to enable positive identification of a stable tertiary structure. It should be noted though, that alpha helical proteins generally display much less chemical shift dispersion than proteins containing beta sheets, especially in the absence of aromatic residues, which is the case with these Par-4 fragments. Nevertheless, we went on to investigate the 215-332 construct for further evidence of structure, reasoning that the extra 25 amino acids may assist in folding. However, the 1D NMR spectrum appears similar to the 240-332 spectrum **(Figure 3(b)).** In addition, a low concentration 1D NMR spectrum at pH 7.3 was acquired using the 215-332 fragment **(Figure 3(c)).** This spectrum, despite increased noise due to low concentration, is similar to the low pH spectra, further confirming that there is little change of structure at acidic pH.

Task 2 Determine the structure of the Par-4 CTR via heteronuclear multi-dimensional NMR

This task requires prior identification of conditions for a solubility of the Par-4 CTR to 3 mg/ml or so. Since under the conditions tested, all of the above constructs yield protein of limited solubility, we reasoned that the extreme C-terminus of Par-4 may be responsible for the solubility problems. The last 3 turns of the putative leucine zipper helix contain two hydrophobic residues, in positions "e" and "g". Since hydrophobic residues at "e" and "g" positions are indicative of higher order oligomerization, these residues could be responsible for the observed aggregation at high concentrations. Therefore, we are preparing constructs in which the last 3-6 turns of the putative Par-4 helix are deleted. Solubility and ability to specifically bind atypical PKCs (see below) will be tested.

Task 3 Define the minimal region of WT1 necessary and sufficient for interaction with Par-4 CTR (NOTE: Task 3 is to be accomplished simultaneously to task 2)

Since the interaction between Par-4 and the atypical PKCs has gained wide acceptance as a mode of action of Par-4, we have chosen to focus upon that task. Thus, we skip to task 4.b.

Task 4.b Begin structural determination of a complex between the Par-4 CTR and a ZF-containing region of an aPKC or other Par-4 interacting proteins

a. Express unlabeled aPKC Zn-binding domain [month 25-26]

PKC-zeta and PKC-lambda/iota Zn-binding domain constructs in pGEX-2TK (gifts from J. Moscat) were transformed into *E. coli* strains BL21, BL21(DE3), JM101 and DH5α, but very little fusion protein could be expressed (**Figure 4(a)**). A check of the sequence revealed the presence of eight rare Arginine and one rare Leucine codon in a stretch of 58 amino acids in the PKC zeta Zn-binding domain (and a similar ratio in PKC lambda/iota). Expression from BL21(DE3) codon plus RIL series cells (Stratagene, La Jolla, CA) which are engineered to produce the rare codon tRNAs, improved expression significantly (greater than 20 mg/L and 10 mg/L growth for PKC-zeta and PKC-lambda/iota respectively, **Figure 4(b)**). When induced at 37°C, at least 95% of the protein was in the pellet after sonication. Induction at 20°C increased solubility to above 95%, albeit at a slightly lower yield (15 mg/L). Addition of 500 μM $ZnSO_4$ to the LB media (5) (6) improved yield by another 20%.

b. Determine oligomerization state via gel filtration and NMR diffusion [month 26-27]

Gel filtration (**Figure 5**) clearly shows that the PKC zeta ZBD produced as above is monomeric under the conditions tested.

c. Optimize buffering conditions (pH, buffer, salt, temperature) [month 26-30]

We are currently optimizing conditions for purification and fusion cleavage. We have also begun GST and MBP pulldowns between the Par-4 and aPKC domains in order to verify that we have the "active" conformations. Preliminary results suggest a high affinity interaction, but further controls need to be performed. If either of the domains is present as a mixture of unfolded and folded populations, pull-downs may be useful to select the folded fraction.

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KEY RESEARCH ACCOMPLISHMENTS

- subcloned, expressed and isolated various Par-4 CTR fragments
- Par-4 CTR is highly alpha helical
- Par-4 CTR has a tendency to aggregate and precipitate at high concentration
- acidic pH/high salt increases solubility somewhat
- CD and NMR spectra confirm that acidic pH/high salt does not significantly alter the structure of Par-4 CTR

- subcloned, expressed and isolated the PKC zeta ZBD
- subcloned and expressed the PKC lambda/iota ZBD
- gel filtration suggests that the PKC zeta ZBD is monomeric at under the conditions tested

REPORTABLE OUTCOMES

The above outcomes should be considered as preliminary findings setting the table for further structural studies.

CONCLUSIONS

The self-associative properties of the Par-4 CTR, together with the monomeric character of the PKC zeta ZBD suggest several avenues for future research. It is possible that the Par-4 CTR becomes more soluble when in the presence of the PKC zeta ZBD, thus, we are preparing to combine these fragments, and test for total solubility and size of any complexes formed. Also, as mentioned previously, shorter Par-4 constructs may increase solubility even in the absence of PKC fragments. Further mutagenesis may be attempted, in an effort to identify other highly soluble fragments which are capable of binding aPKCs. If no conditions can be found for which an appropriate Par-4 fragment is soluble to close to millimolar concentration, it should still be possible to map the region of the PKC zeta ZBD which interacts with the Par-4 aggregates, by titrating Par-4 into a PKC zeta sample and monitoring changes in the NMR spectra.

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APPENDICES

ACRONYM AND SYMBOL DEFINITIONS

1D, 2D, 3D, 4D NMR	one, two, three, four-dimensional NMR
3C pro	Human Rhinovirus-14 3C protease
CTR	Carboxy-Terminal Region
GST	Glutathione S-Transferase
LZ	Leucine Zipper
MIR	Minimal Interacting Region
par-4	Prostate Apoptosis Response #4 (gene)
Par-4	Prostate Apoptosis Response #4 (protein)
Par-4 CTR	Par-4 Carboxy-Terminal Region (residues 268-332)
PKC	Protein Kinase C
WT1	Wims' Tumor suppressor protein

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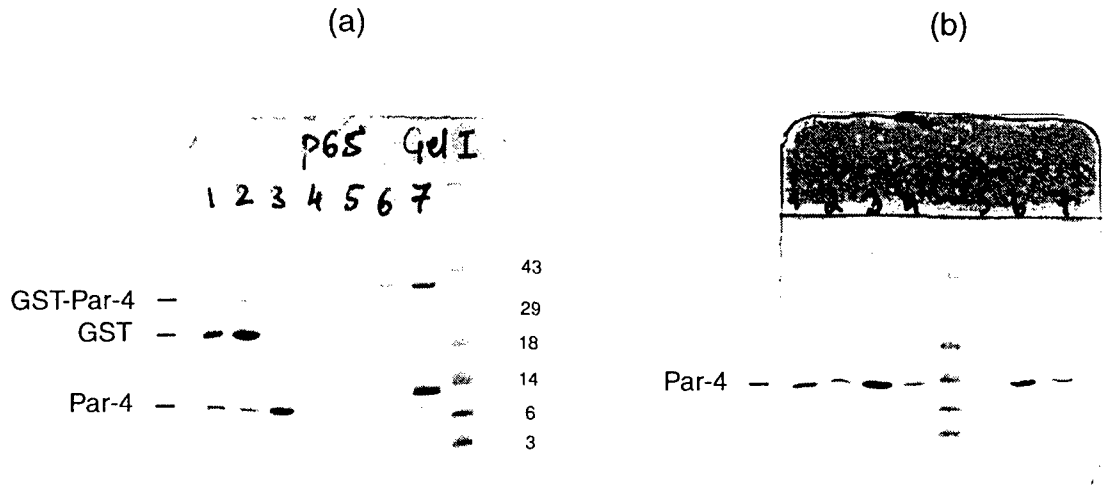

Figure 1. SDS PAGE of Par-4 CTR fragments.

gel (a)

Lanes 1,2: GST-Par-4 240-332 bound to glutathione resin, then cut by 3C pro

Lane 3*-4: Supernatant after centrifugation of resin from Lane 1,2

Lane 5-7*: pMAL-Par-4 215-332 bound to amylose resin, eluted by maltose, and cut by Factor Xa

Lane 8: Molecular Weight Markers

gel (b): Various aliquots of purified Par-4 215-332 fragment after cleavage from GST by 3C pro.

Lane 1-3: Concentrated solutions at low pH. The concentrated solution (0.55 mg/ml, pH 2.5) sampled in Lane 3 was used to create the low pH sample used for CD and NMR spectra (Fig. 2 and 3).

Lane 5-7*: Assay of Par-4 fragment washed from glutathione resin after 3C pro cleavage using (Lane 5) 150 mM NaCl, (Lane 6)1M NaCl, and (Lane 7) a second 1M NaCl wash.

Between Lane 4 and 5: Molecular Weight Markers

* In order to increase sensitivity, 10 μL of StrataClean resin (Stragene, La Jolla CA), a non-specific protein binder, was added to the samples for Lanes (a)3, (a)7 and (b) 5-7. After centrifugation and removal of supernatant, 10 μL of 2X SDS buffer was added to the Strataclean beads, and this mixture was loaded onto the gel.

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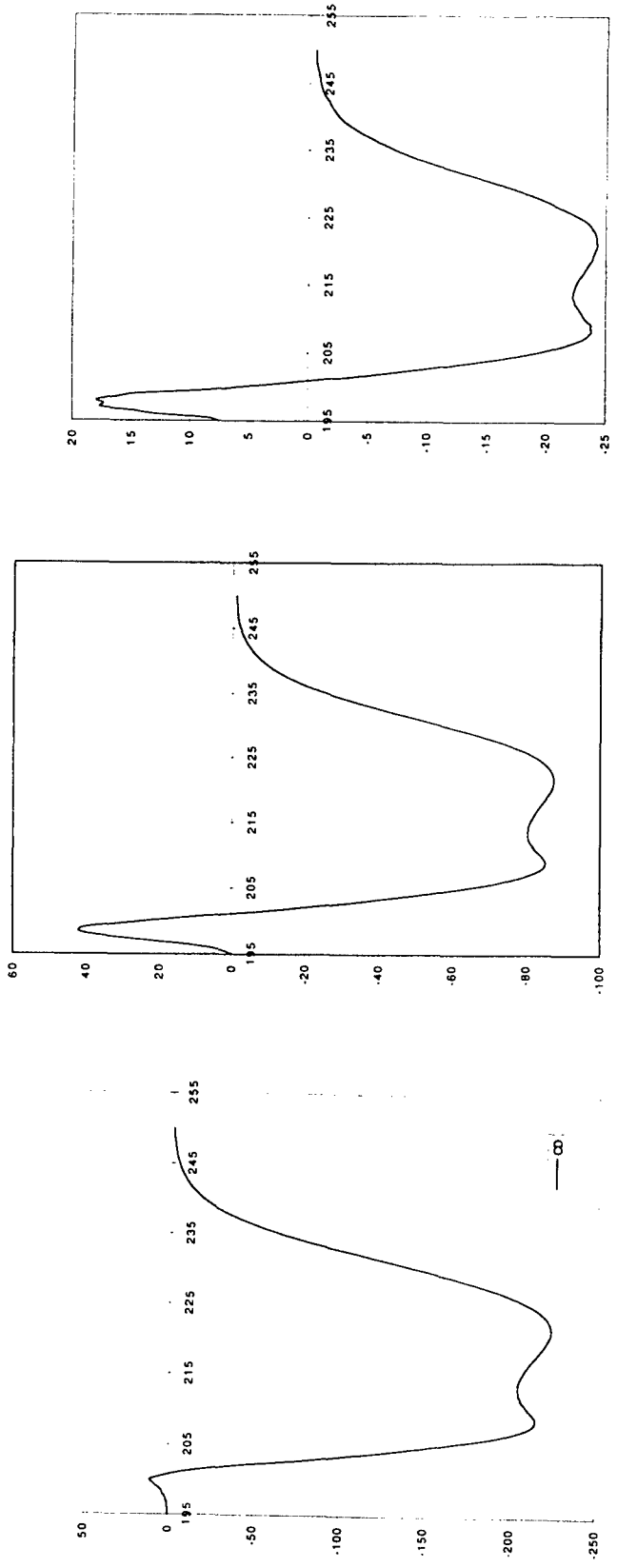

Figure 2. CD spectra, in 12 mM Sodium Phosphate buffer and 500 mM NaCl, of the Par-4 240-332 fragment at a concentration of (a) 0.26 mg/ml at pH 2.69 (b) 0.15 mg/ml at pH 3.65 (c) less than 0.05 mg/ml at pH 7.3.

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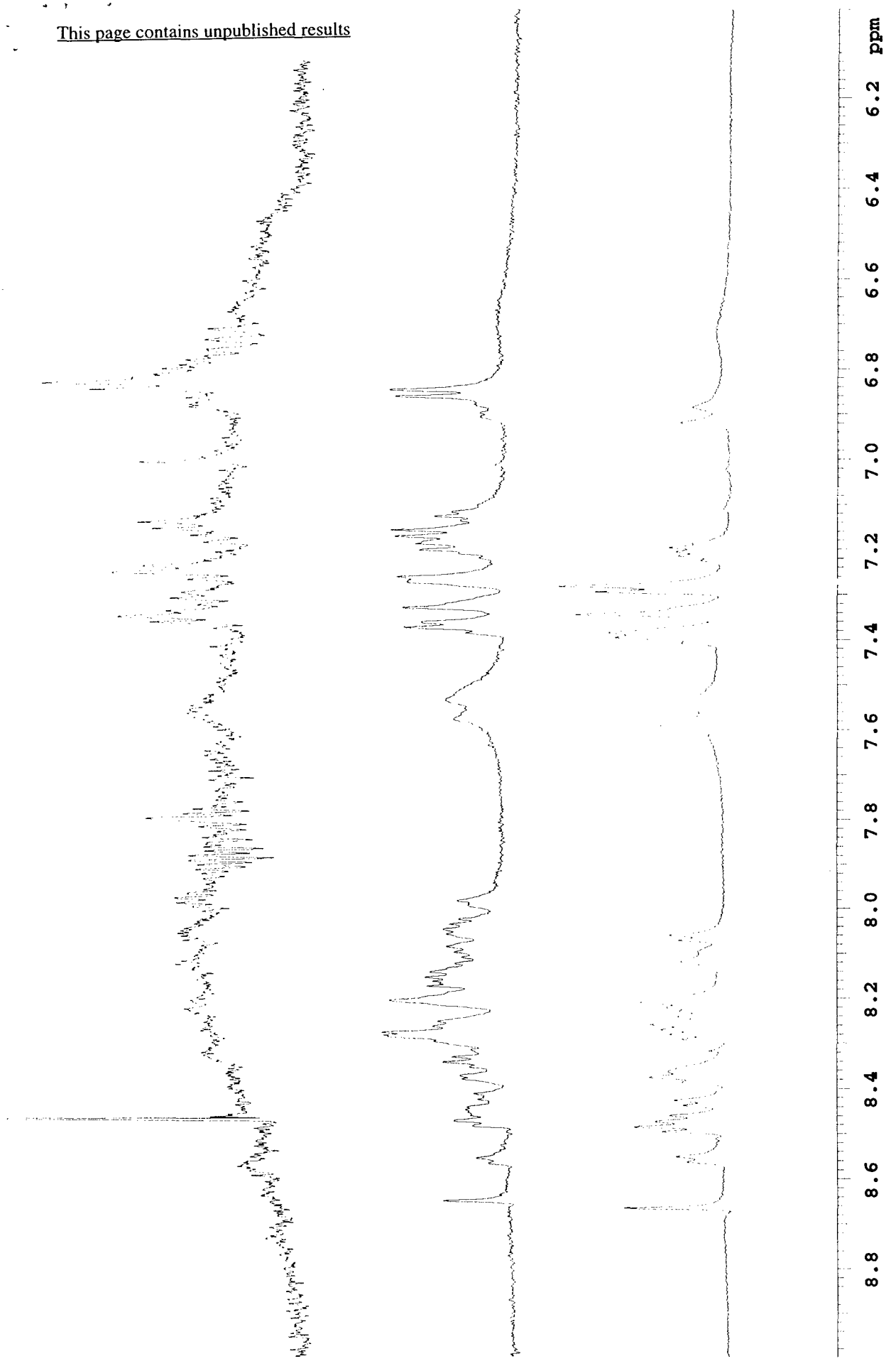

Figure 3. Downfield region of one dimensional ^{1}H NMR spectra of the Par-4 CTR taken at 600 MHz and 30°C: (a) 0.55 mg/ml Par-4 240-332 fragment, pH 3.5, 2416 transients acquired. (b) 0.55 mg/ml Par-4 215-332 fragment, pH 2.5, 512 transients. (c) 0.04 mg/ml Par-4 215-332 fragment, pH 7.3, 18000 transients.

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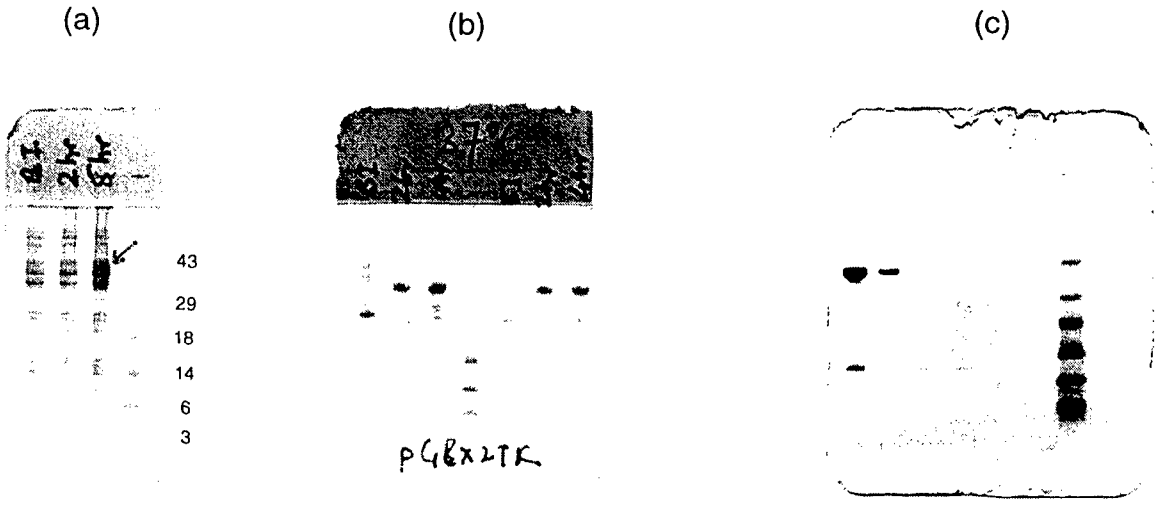

Figure 4. SDS PAGE of PKC zeta Zn-binding domain.

gel (a) GST fusion construct transformed into BL21(DE3) and grown in LB media

Lane 1: Before Induction
Lane 2: 2 hours after induction by IPTG
Lane 3: 8 hours after induction by IPTG
Lane 4: Molecular Weigth Markers

gel (b): GST fusion construct transformed into BL21(DE3) codon plus RIL and grown in LB media

Lane 1: Before Induction
Lane 2: 2 hours after induction by IPTG
Lane 3: 4 hours after induction by IPTG
Lane 4: Molecular Weigth Markers
Lane 5-7: Same as Lanes 1-3

gel (c): Size Exclusion Column (SEC) Results (see Figure 5):
MBP fusion after partial purification by amylose resin, elution by maltose, and cleavage by thrombin

Lane 1: Input to SEC
Lane 2: Fraction 18 (MBP)
Lane 3: Fraction 23 (PKC zeta Zn-binding domain)
Lane 4*: Fraction 27
Lane 5*: Fraction 33
Lane 6*: Fraction 35
Lane 7: Molecular Weigth Markers

* 10 µL of StrataClean resin was added to the samples for Lanes (c) 4-6 as discussed in the legend to Figure 1.

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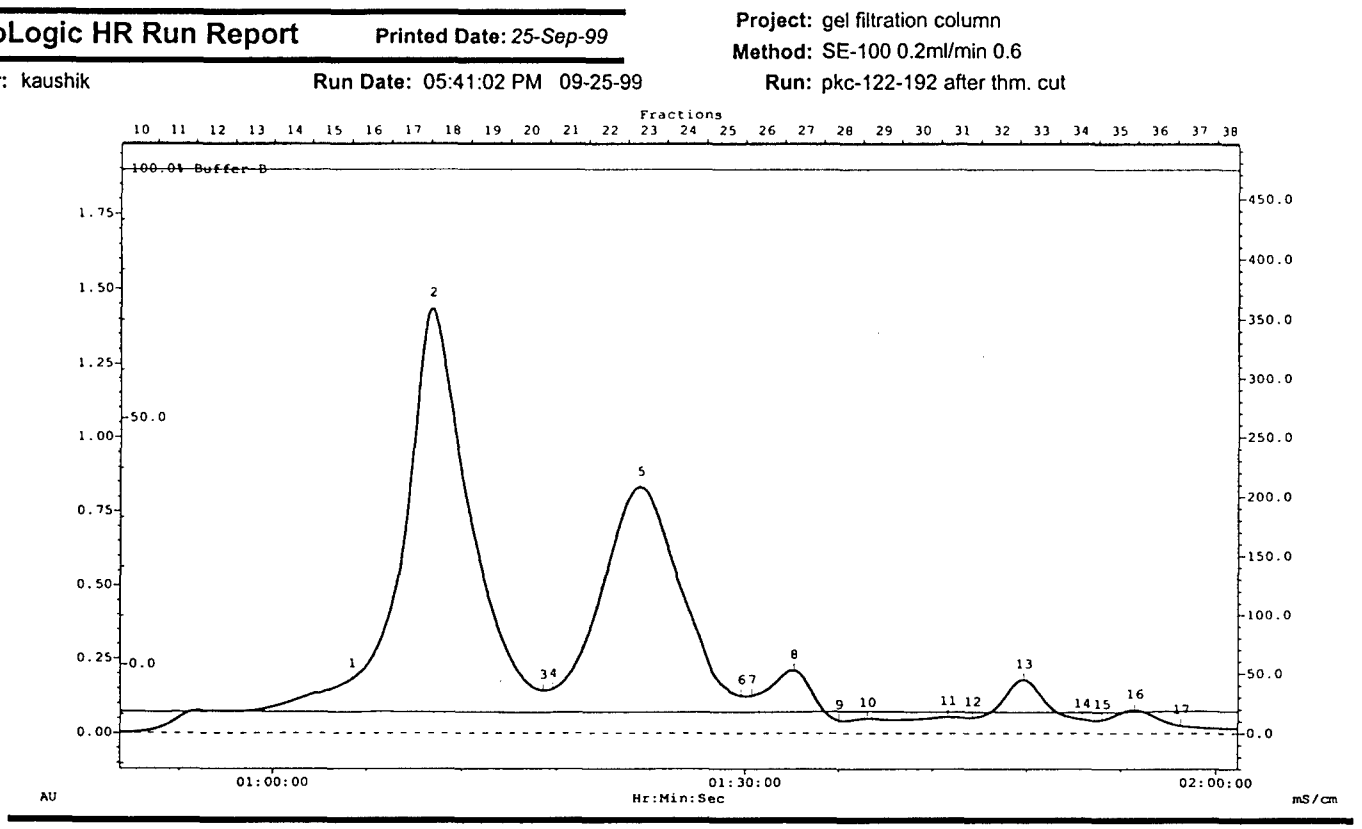

Figure 5. Size Exclusion Chromatography (SEC) of the MBP/PKC zeta Zn-binding domain mixture, after partial purification of MBP-fusion by amylose resin, elution with maltose, and fusion cleavage by thrombin. Aliquots from marked peak numbers 2, 5, 8, 13 and 16 were run on SDS PAGE (see Figure 4(c)). A flow rate of 0.2 ml/min was used with a Bio-Prep SE 100/17 column (BioRad, Hercules, CA).

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